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TITLE: CLONING AND EXPRESSION OF GENES FOR DENGUE VIRUS TYPE-2 ENCODED-ANTIGENS FOR RAPID DIAGNOSIS AND VACCINE DEVELOPMENT

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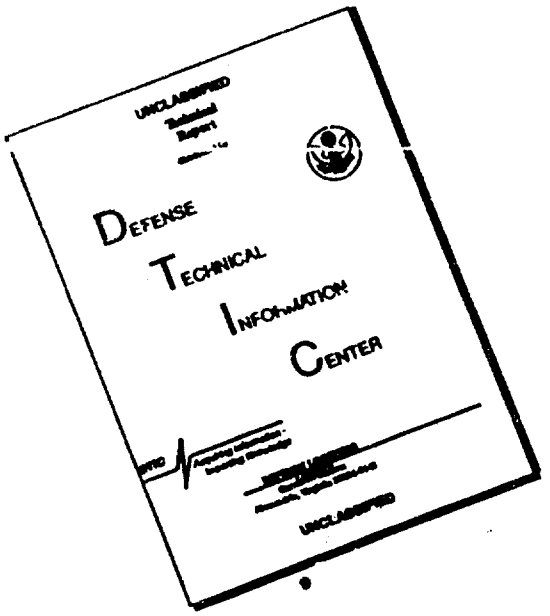

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

1. The complete sequence of dengue virus type 2 (New Guinea strain C) has been determined. The genome is 10,723 nucleotides long. The organization of the genome is similar to that of yellow fever virus.
2. The neutralization epitope of a monoclonal antibody 3H5 has been mapped to a region of 12 amino acids QLKLNWFKKGSS located in E protein of DEN-2 virus. The antibodies raised against this peptide can neutralize the infectivity of the virus, although these results need to be confirmed. The peptide antisera can minick 3H5 in other tests. The peptide also reacts with 3H5 with specificity.
3. We have obtained experimental evidence which demonstrate more directly that the 3'-terminus of DEN-2 RNA has the potential to form secondary structure in solution. This finding has important implications for flavivirus replication.

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FOREWORD

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_X_ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

_X_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

R Ravindranathan 2-26-91

PI Signature Date

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3.0 INTRODUCTION

a. General:

Dengue is a human disease caused by dengue virus, a member of the family flaviviridae (Westaway et al., 1985). Dengue viruses are of four distinct serotypes and are transmitted to humans principally by *Aedes aegypti* mosquitos. Apart from dengue fever (DF), a more severe and fatal form of the disease occurs in children called dengue hemorrhagic fever (DHF) which could lead to the shock syndrome (DSS) (for a review see Halstead, 1988). A potential problem associated with monovalent dengue vaccines is that individuals infected with one serotype are fully susceptible to infection with other serotypes resulting in a serious form of the disease, DHF, or DSS. A live attenuated vaccine was developed, but its use in volunteers was discontinued due to inconsistent neutralizing immune response among some volunteers, (Bancroft et al., 1984; Eckels et al., 1984).

b. Genomic organization

Using recombinant DNA techniques it has been possible to determine the nucleotide sequences of several flavivirus genomes. The first total flavivirus nucleotide (nt) sequence was that of yellow fever virus (YF) (Rice et al., 1985). Since then, the complete or partial nucleotide sequences of a number of flavivirus RNA genomes have been determined: West Nile virus (WN) (Castle et al., 1985; 1986), DEN-1 (Mason et al., 1987), DEN-4 (Zhao et al., 1987; Mackow et al., 1988), PR-159 isolate of DEN-2/S1 strain (Hahn et al., 1988), DEN-2 strain 1409 isolated in Jamaica in 1983 (DEN-2JAM (Duebel et al., 1986; 1988), JE (Sumiyoshi et al., 1987), and Kunjin (Coia et al., 1988) The complete sequence of the DEN-2NGS-C genome, 10,723 nt in length with the exception of about seven nt from the 5'-noncoding region was determined in our laboratory (Yaegashi et al., 1986; Putnak et al., 1988; Irie et al., 1989).

c. Use of recombinant DNA-based expression systems

The amount of virion proteins synthesized in the infected host cells is not sufficient to permit detailed characterization of their biological function in viral/cell interaction, neutralization and host immune response, replication and assembly of virus particles. In order to circumvent this problem, several groups have begun to utilize recombinant DNA-based high level expression systems and characterize functions of flavivirus proteins. Three notable expression systems are: 1) the E. coli expression system (2) the recombinant vaccinia virus system, and (3) the recombinant baculovirus system. Of these three, the first system gives rise to proteins with no posttranslational modifications such as glycosylation, phosphorylation or processing of the polyprotein precursors. Therefore, it is of limited use for the flavivirus proteins which do undergo glycosylation and proteolytic processing. Yet, this system was used in our laboratory due to its simplicity, to map the antigenic determinants of monoclonal antibodies against the DEN-2 E protein, having a high neutralizing titer (Trirawatanapong et al., 1991) (see below). Using this E. coli-based expression system, we also successfully mapped the antigenic domains of the nonstructural protein NS1 (Putnak et al., 1988).

(d) Mapping epitopes

The variable portion of the flavivirus glycoprotein E which is the major portion of the molecule contains epitopes which determine the type specificity and complex specificity. These antigenic specificities form the basis for serological classification of flaviviruses as determined by haemaglutination-inhibition (HI) and neutralization tests. The glycoprotein E is the most important for the induction of neutralizing antibodies as well as protective immunity (Della-Porta and Westaway, 1977; Kitano et al., 1974; Heinz et al., 1981). In order to develop a subunit vaccine against dengue virus, it is important to identify the region of E glycoprotein which interacts with the host cell, or to identity its neutralizing epitopes.

Heinz et al. (1983a) presented a model for the antigenic structure of flavivirus glycoprotein E consisting of variable and conserved epitopes by analysis of glycoprotein of

tick-borne encephalitis virus (TBE) (Heinz et al., 1983a; 1983b). These studies revealed the existence of three antigenic domains (A,B,and C) on E glycoprotein of TBE. Domains A & B contain neutralization-protective, and HI epitopes. The 3-dimensional structure of the E protein is important for the integrity of these determinants. Flavivirus group-reactive monoclonal antibodies although reactive in HI, were not involved in neutralization (Heinz, et al., 1983b). Heinz also noted that only those monoclonal antibodies (Mabs) which neutralized the virus in vitro also passively protected mice against lethal challenge with TBE virus.

Various methods are available to determine the precise location of antigenic sites on protein molecule (Berzofsky, 1985). One approach involves the use of Mabs as specific reagents for defining single epitopes on the complex antigenic structure of protein molecules. Using methods involving screening of purified proteolytic fragments of a protein molecule and/or screening a collection of overlapping synthetic peptides, the antigenic determinants of a number of viral glycoproteins have been studied (Roehrig et al., 1982; 1983; Lubeck and Gerhard, 1982; Emini et al., 1982; Volk et al., 1982; Massey and Schochetman, 1981; Mathews and Roehrig, 1984; Mehra et al., 1986). Second approach used was by selecting escape variants against neutralizing Mabs, and sequence the variants in the region of the genome encoding E and M (Lobigs et al., 1987). It was found that each of the variant resulted from a single nt change in the E protein coding sequence leading to a nonconservative amino acid substitution at position 71 or 72 in the N-terminal region of E. Third approach made use of the lgt 11 expression system, which involves construction of expression libraries containing fragments of gene encoding the antigen, and sequence the cDNA expression clone that reacts with an antibody. The DNA sequence encoding the epitope is attributed to sequences that are shared by multiple antibody-positive recombinant clone (Mehra et al., 1986). We developed another useful approach for mapping the linear antigenic determinant of E protein of DEN-2 recognized

by a type-specific and neutralizing Mab, 3H5, which has been shown in previous studies to have a significantly high neutralization titer (Gentry et al., 1982).

(e) Presence of a stable secondary structure at the 3'-end of flavivirus genomes

Interestingly, the potential to form a stable secondary structure is highly conserved among the various flavivirus RNA molecules, as deduced from the primary sequence data and the computer-derived energy calculations (Tinoco et al., 1973). Two lines of experimental evidence suggested that such secondary structures could exist in solution. First, Brinton et al. (1986) found that nucleotides within the putative region of secondary structure were partially resistant to ribonuclease. Second, Hahn et al., (1987) reported the isolation of rare cDNA clones of DEN-2 RNA (S1/candidate vaccine strain of PR-159 isolate) as well as that of MVE RNA, which could have arisen by self-priming of the 3'-terminal base-paired region during reverse transcription.

In our laboratory, we devised a novel method to generate radiolabeled transcripts of high specific activity in vitro, which contain truncated 3'-terminal sequences of DEN-2 RNA (New Guinea-C strain) identical in sequence to the genomic RNA. Such transcripts were used to investigate the possible formation of secondary structure at the 3'-terminus. By digesting with RNase A, three protected fragments of 16-23 nucleotides in length were obtained. These RNase-resistant fragments were mapped within the 3'-terminal 96 nucleotide region by analyzing their distinct susceptibilities to RNase H digestion, subsequent to the formation of RNA:DNA hybrids with synthetic oligodeoxynucleotides of known sequence. These results strongly support that such stable secondary structures are indeed formed in solution.

4.0 Body of the report

4.1 Complete nucleotide sequence determination of DEN-2 RNA genome

In the Final Report submitted on March 1, 1986, we described the work done supported by the USAMRDC Contract (DAMD 17-82-C-2051). At that time, we had determined only 4586 nucleotides of DEN-2 genome by cDNA cloning and sequence analysis. Subsequently, the complete sequence analysis of the genome was accomplished. The genome of DEN-2 virus (New Guinea-C strain) is 10,723 nucleotides in length. It has one long open reading frame coding for a polyprotein of 3391 amino acids in length, which is processed into three structural and seven nonstructural proteins by cellular and viral proteinase(s). The cloning and sequence analysis of the entire DEN-2 genome was reported in the **ANNUAL REPORT dated October 31, 1988**, and was published (Putnak et al., 1988; Irie et al., 1989).

4.2 Expression of dengue viral antigens in E. coli

One of the objectives of the Contract is to express the dengue viral antigens in E. coli to produce large amounts of the protein and to produce polyclonal antibodies against these antigens. We used different cDNA clones for expression in E. coli using the pMR100 vector. Different epitopes of dengue viral antigens were expressed as fusion to the E. coli β-galactosidase (E. coli β-Gal). Two advantages of this expression system are that (1) it is easy to identify the recombinants, and (2) the fusion proteins can be purified to near homogeneity in a single step using an affinity chromatography based on binding to a substrate analogue of E. coli β-Gal. (1) Using this system, we first expressed the E and NS1 epitopes, as these two proteins are important for the development of vaccines against DEN-2 virus. We learnt that the fusion proteins containing the hydrophobic C-terminal regions of E protein, instead of being larger than E. coli β-Gal, undergo degradation in E. coli to a limit size which was less than the authentic E. coli β-Gal. The fusion proteins were synthesized in abundant quantities and could be readily purified to near homogeneity in a single step affinity chromatography as expected. (2) The C-terminal domain of NS3 antigen of DEN-2 was also expressed in E. coli as a fusion protein to E. coli β-Gal using the cDNA clone YS505 (Irie et al., 1989). For the expression of NS5, the cDNA clone A4 was

used. Analysis of the fusion proteins by Western blots, showed that the fusion proteins were unstable in E. coli and underwent degradation. These studies showed that the expression of DEN-2 antigens as fusion proteins to E. coli β-Gal did not give rise to proteins of expected sizes due to degradation, and the immunoreactivities of these fusion proteins to the well-characterized monoclonal antibodies against DEN-2 antigens were aberrant. The antibodies raised these fusion proteins in rabbits were also found to be not immunoreactive to the native viral antigens. Therefore, it seems that expression of DEN-2 viral antigens in E. coli must be carried out as unfused proteins, and probably only as hydrophilic regions of the proteins. The details of these expression studies are described in the **ANNUAL REPORT dated November 17, 1987**.

4.3 Mapping of the neutraiization epitope of DEN-2 E protein

One of the specific aims of the Contract proposal is to express the antigens coded by the dengue virus type 2 in order to understand their functions, and develop a safer vaccine to control dengue infections. Heinz et al. (1983) showed that only those monoclonal antibodies which neutralized the virus in vitro also passively protected mice against lethal challenge with tick-borne encephalitis virus. Lobigs et al., (1987) defined the antigenic determinant involved in the neutralization of YF on E protein. The approach used was to select neutralization escape variants against two neutralizing monoclonal antibodies, and sequence the variants in the region of the genome encoding E and M. It was found that each of the variant resulted from a single nucleotide change in the E region leading to a non-conservative amino acid substitution at position 71 or 72. Thus, the antigenic determinant (s) involved in the neutralization are localized in the E protein. Therefore, it was important to express E protein to analyze its antigenic determinants.

We chose pOTS expression vector for producing unfused E protein of DEN-2 in E. coli because previous studies mentioned above showed that the dengue viral fusion proteins (to E. coli β-Gal) were unstable in E. coli. Moreover, the synthesis of the antigen

of interest can be tightly controlled in this system. The vector contains the strong λP_L promoter, and expression of any gene cloned under the control of this promoter can be induced with temperature. The host is E. coli N5151 which produces thermo-labile λ repressor, which is inactivated at 42 C. The expression of nearly full-length E protein, or the truncated polypeptides were tested for their imuunoreactivity by Western blotting technique using the polyclonal hyper mouse ascitis fluid (HMAF), and the monoclonal 3H5 antibody. First, the epitope of 3H5 antibody on E protein was localized within a 180 amino acid residue region between two BamHI sites within the coding region of E protein. Subsequently, more targeted deletions within the 180 amino acid region were made using the polymerase chain reaction (PCR). A hydrophilic region encoded by QLKLNWFKKGSS (386-397) was mapped to be the 3H5 reactive site on the E protein.

The following experimental evidence indicated that this region of E protein represents the 3H5-reactive site. (1) The antibodies raised against a synthetic peptide containing 12 amino acids mimicked 3H5 in immunoreactivity with the peptide as shown by ELISA. The anti- peptide antibody reacted specifically with the peptide at the dilution of 1:10,000, when compared with preimmune sera. Similarly, the reactivity of 3H5 monoclonal antibody to peptide also showed specificity when compared with unrelated monoclonal antibodies 3E9 and A16, which are monoclonal antibodies against DEN-2 NS1 protein and Herpes virus glycoprotein, respectively. (2) The anti-peptide antibodies reacted with the E protein of DEN-2 virus in vitro. For this experiment, the CV1 cells were infected with DEN-2 virus, and labeled the proteins using ^{35}S methionine. Immunoprecipitation of labeled proteins from the infected cells, followed by SDS/PAGE indicated that anti-peptide antibody recognized a protein with a mobility in gel similar to that of gp 60 immunoprecipitated with either HMAF or 3H5 monoclonal antibody. These results are described in the **ANNUAL REPORTS dated December 11, 1989 and December 12, 1990**.

4.4 Detection of stable secondary structure at the 3'-terminus of dengue virus type 2 RNA

The 3' terminal sequences of approximately 100 nucleotides of flavivirus genomes have been suggested to have a highly conserved secondary structure, based on predictions from the known nucleotide sequence data, and free energy calculations using computer programs (Tinoco et al., 1973). Two lines of experimental evidence suggested that such secondary structures could exist in solution. First, Brinton et al. (1986) found that nucleotides within the putative region of secondary structure were partially resistant to ribonuclease. Second, Hahn et al., (1987) reported the isolation of rare cDNA clones of DEN-2 RNA (S1/candidate vaccine strain of PR-159 isolate) as well as that of MVE RNA, which could have arisen by self-priming of the 3'-terminal base-paired region during reverse transcription. In order to test the existence of secondary structure in solution, we devised a strategy to generate truncated RNA molecules from about 0.3 to 1.4 kilobases in length in vitro, having the same polarity and nucleotide sequence as dengue virus type 2 (DEN-2) RNA (NEW Guinea-C strain). When these labeled RNA molecules were digested by RNase A, and analyzed by denaturing polyacrylamide gel electrophoresis, three protected fragments of 16, 20, and 23 nt in length were reproducibly obtained. To examine whether these RNase A-resistant fragments emerged from a stable secondary structure formed in solution consisting of 3' terminal sequences, hybridization of the RNase A-resistant fragments to four chemically synthesized oligodeoxynucleotides, complementary to nt 1-24, 25-48, 49-72, and 73-96 from the 3'-terminus of DEN-2 RNA, followed by RNase H digestion were carried out. Two of the four oligodeoxynucleotides were sufficient to render all three RNase A-resistant fragments susceptible to RNase H digestion. From these data, it is clear that a stable secondary structure is formed in which the nucleotides 18- 62 from the 3'-terminus are very likely to be involved, and in addition, it is possible to deduce the RNase A cleavage sites. The experimental evidence for the formation of stable secondary structure at the 3'-terminus of DEN-2 RNA, and the

potential use of these unique transcripts to identify the viral and/or host proteins which might interact at the 3'-terminus of DEN-2 RNA during initiation of replication are described in the **ANNUAL REPORT dated December 11, 1989.** A manuscript describing these details has been accepted recently in GENE (1991).

5.0 Literature Cited

Bancroft, W.H., Scott, R.M., Eckels, K.H., Hoke, C.H., Simms, T.E., Jesrani, K.D.T., Summers, P.L., Dubois, D.R., Tsoulos, D., and Russell, P.K. (1984). Dengue virus type 2 vaccine: reactogenecity and immunogenecity in soldiers. J. Infect. Diseases 149, 1005-1010.

Berzofsky, J.A. (1985). Intrinsic and extrinsic factors in protein antigenic structure. Science 229, 932-940.

Brinton, M.A., Fernandez, A.V., and Dispoto, J.H. (1986). The 3'-nucleotides of flavivirus genome RNA form a conserved secondary structure. Virology 153, 113-121.

Castle, E., Leidner, U., Novak, T., Wengler, G., and Wengler, G. (1986). Primary structure of the West Nile flavivirus genome coding for all non-structural proteins. Virology 149, 10-26.

Castle, E., Novak, T., Leidner, U., Wengler, G., and Wengler, G. (1985). Sequence analysis of West Nile virus and of the genome sequence for these proteins. Virology 145, 227-236.

Coia, G., Parker, M.D., Speight, G., Byrne, M.E., and Westaway, E.G. (1988). Nucleotide and complete amino acid sequence of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. J. Gen. Virol. 69, 1-21.

Della-Porta, A.J., and Westaway, E.G. (1977). Immune response in rabbits to virion and non-virion antigens of the flavivirus Kunjin. Infect. Immun. 15, 874-882.

Duebel, V., Kinney, R.M., and Trent, D.W. (1988). Nucleotide sequence and deduced amino acid sequence of the nonstructural proteins of dengue type 2 virus, Jamaica genotype: comparative analysis of the full-length genome. Virology 165, 234-244.

Deubel, V., Kinney, R.M., and Trent, D.W. (1986). Nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue type 2 virus, Jamaica genotype. Virology 155, 365-377.

Eckels, K.H., Scott, R.M., Esposito, J.J., Brown, J., Dubois, D.R., Summers, P.L., Russell, P.K. and Halstead, S.B. (1984). Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells. Am. J. Trop. Med. Hyg. 33, 684-689.

Emini, E.A., Jameson, B.A., Lewis, A.J., Larsen, G.R., and Wimmer, E. (1982). Poliovirus neutralization epitopes: analysis and localization with neutralizing monoclonal antibodies. J. Virol. 43, 997-

Gentry, M.K., Henchal, E.A., McCown, J.M., Brandt, W.E., and Dalrymple, J.M. (1982). Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am J. Trop. Med. Hyg. 31, 548-555.

Hahn, C.S., Hahn, Y.S., Rice, C.M., Lee, E., Dalgarno, L., Strauss, E.G. and Strauss, J.H. (1987). Conserved elements in the 3'-untranslated region of flavivirus RNAs and potential cyclization sequences. J. Mol. Biol. 198, 262-267.

Hahn, Y.S., Galler, R., Hunkapillar, T., Dalrymple, J.M., Strauss, J.H., and Strauss, E.G. (1988). Nucleotide sequence of dengue 2 RNA and comparison of the encoded proteins with those of other flaviviruses. Virology 162, 167-180.

Halstead, S.B. (1988). Pathogenesis of dengue: Challanges to molecular biology. Science 239, 476-481.

Heinz, F.X., Tuma, W., and Kunz, C. (1981). Antigenic and immunological properties of defined physical forms of tick-borne encephalitis virus structural proteins. Infect. and Immun. 33, 250-257.

Heinz, F.X., Berger, R., Tuma, W., and Kunz, C. (1983a). A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology 126, 525-537.

Heinz, F.X., Berger, R., Tuma, W., and Kunz, C. (1983b). Location of immunodominant antigenic determinants on fragments of the tick-borne encephalitis virus glycoprotein: Evidence for two different mechanisms by which antibodies mediate neutralization and haemagglutination inhibition. Virology 130, 485-501.

Irie, K, Mohan, P.M., Sasaguri, Y., Putnak, R. and Padmanabhan, R. (1989). Sequence analysis of cloned dengue virus type 2 genome(New Guinea-C strain) Gene.75, 197-211.

Kitano, T., Suzuki, K., and Yamaguchi, T. (1974). Morphological, chemical, and biological characterization of Japanese encephalitis virus virion and its hemagglutinin. J. Virol. 14, 631-639.

Lobigs, M., Dalgarno, L., Schlesinger, J.J., and Weir, R.C. (1987). Location of a neutralization determinant in the E protein of yellow fever virus (17D vaccine strain). Virology 161, 474-478.

Lubeck, M., and Gerhard, W. (1982). Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. Virology 118, 1-

Mackow, E., Makino, Y., Zhao, B., Zhang, Y-M., Markoff, L., Buckler-White, A., Guiler, M., Chanock, R., and Lai, C-j. (1987). The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins. Virology 159, 217-228.

Mohan, P.M. and Padmanabhan, R. (1991). Detection of stable secondary structure at the 3-terminus of dengue virus type 2 RNA. Gene, In Press.

Mason, P.W., McAda, P.C., Mason, T.L., and Fournier, M.J. (1987). Sequence of the dengue virus genome in the region encoding the three structural proteins and the major nonstructural protein NS1. Virology 161, 262-267.

Massey, R.J., and Schochetman, G. (1981). Topographical analysis of viral epitopes using monoclonal antibodies: mechanism of virus neutralization. Virology 115, 20-.

Mathews, J.H., and Roehrig, J.T. (1984). Elucidation of the topography and determination of the protective epitopes on the E glycoprotein of Saint Louis encephalitis virus by passive transfer with monoclonal antibodies. J. Immunol. 132, 1533-1537.

Mehra, V., Sweetser, D., and Young, R.A. (1986). Efficient mapping of protein antigenic determinants. Proc. Nat. Acad. Sci. USA 83, 7013-7017.

Putnak, R., Charles, P.C., Padmanabhan, R., Irie, K., Hoke, C., and Burke, D. S. (1988). Functional and antigenic domains of the dengue-2 virus non-structural glycoprotein NS1. Virology 163, 93-103.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L. and Strauss, J.H. (1985). Nucleotide sequence of yellow fever virus: Implications for flavivirus gene expression and evolution. . Science 229, 726-733.

Roehrig, J.T., Day, J.W., and Kinney, R.M. (1982). Antigenic analysis of the surface glycoproteins of a Venezuelan equine encephalomyelitis virus (TC-83) using monoclonal antibodies. Virology 118, 269-.

Roehrig, J.T., Mathews, J.H., and Trent, D.W. (1983). Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology 128, 118-.

Sumiyoshi, H., Mori, C., Fuke, I., Morita, K., Kuhara, S., Kondou, J., Kikuchi, Y., Nagamatu, H., and Igarashi, A. (1987). Complete nucleotide sequence of Japanese encephalitis virus genome RNA. Virology 161, 497-510.

Tinoco Jr., I., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. and Gralla, J. (1973). Estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. 246, 40-41.

Volk, W.A., Snyder, R.M., Benjamin, D.C., and Wagner, R.R. (1982). Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus: comparative neutralizing activity. J. Virol. 42, 220-.

Westaway, E.G., Brinton, M. A., Gaidamovich, S.Y., Horzinek, M.C., Igarashi, A., Kaariainen, L., Lvov, D.K., Porterfield, J.S., Russell, P.K. and Trent, D.W. (1985). Flaviviridae. Intervirology. 24, 183-192.

Yaegashi, T., Vakl...a, V.N., Page, K., Sasaguri, Y., Feighny, R., and Padmanabhan, R. (1986). Partial sequence analysis of cloned dengue virus type 2 genome. Gene 46, 257-267.

Zhao, B., Mackow, E., Buckler-White, A., Markoff, L., Chanock, R.M., Lai, C-J, and Makino, M. (1986). Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. Virology 155, 77-88.

6.0 List of Publications Resulting from the USAMRDC Contract

1) Yaegashi, T., Vakharia, V.N., Page, K., Sasaguri, Y., Feighny, R., and Padmanabhan, R. (1986). Partial sequence analysis of cloned dengue virus type 2 genome. Gene 46, 257-267.

2) Henchal, E.A., Narupiti, S., Feighny, R., Padmanabhan, R., and Vakharia, V.N. (1987). Detection of dengue virus RNA using nucleic acid hybridization. J. Virol. Methods 15, 187-200

3) Putnak, R., Charles, P.C., Padmanabhan, R., Irie, K., Hoke, C., and Burke, D. (1988). Functional and antigenic domains of the dengue-2 virus nonstructural glycoprotein NS1 Virology 163, 93-103.

4) Olson, K., Blair, C., Padmanabhan, R., and Beaty, B. (1988). Detection of dengue virus type 2 virus in Aedes albopictus by nucleic acid hybridization with strans-specific RNA probes. J. Clinical Microbiol. 26, 579-581.

5) Irie, K, Mohan, P.M., Sasaguri, Y., Putnak, R. and Padmanabhan, R (1989). Sequence analysis of cloned dengue virus type 2 genome(New Guinea-C strain) Gene.75, 197-211.

6) Mohan, P.M. and Padmanabhan, R. (1991) Detection of stable secondary structure at the 3'-terminus of dengue virus type 2 RNA. Gene, In Press.

7) Trirawatanapong, T., Balachandran, N., Putnak, R., and Padmanabhan, R. (1991). Identification of a neutralization determinant of dengue virus type 2 glycoprotein E. Manuscript to be submitted to Gene.

Abstracts of papers presented at national and international meetings:

1. Padmanabhan, R., Yaegashi, T., Vakharia, V.N., Fieghny, R. (1986). Structural analysis of dengue virus type-2 genome. UCLA symposia on Molecular and Cellular Biology: Positive Strand RNA viruses (April 20-26, 1986; Keystone, CO), Abs. # C1 50, J. Cell. Biochem. Supp. 10D, pp286.

2. Padmanabhan, R., Irie, K., Sasaguri, Y., Trirawatanapong, T., Page, K., Mohan, P.M., Charles, P., Putnak, R., and Burke, D. S. Structural and functional analysis of dengue virus type 2 genome. Presented at the Scientific meeting of the World Health Organization Programme for Vaccine Development on Current Approaches for the development of dengue vaccines" held in Edmonton, Canada (Aug. 7-8,1987).

3. Putnak, R., Charles, P., Irie, K., Padmanabhan, R., Hoke, C., Burke, D. S. Molecular Biology of Dengue Virus NS1 protein; presented at the VIIth International Congress of Virology, Edmonton, Canada; Aug.9-14, 1987.

4. Irie, K., Mohan, P.M., Sasaguri, Y., Putnak, R., Burke, D.S., and Padmanabhan, R. Nucleotide sequence analysis of dengue 2 RNA (New Guinea strain). Presented at the First Asia-Pacific Congress of Medical Virology held at Singapore (November 6-11, 1988) Abs. pp 34.

5. Padmanabhan, R., Thaweesak, T., Padmanabhan, R.V., Burke, D.S., and Putnak, R. Mapping of functional domains of the dengue-2 virus non-structural protein NS1. Presented at the First Asia-Pacific Congress of Medical Virology held at Singapore (November 6-11, 1988) Abs. pp. 195.

6. Trirawatanapong, T., and Padmanabhan, R. Identification of a neutralizing epitope on the envelope glycoprotein of dengue virus type 2 using specific monoclonal antibodies

and the polymerase chain reaction. Presented at the World Health Organization satellite meeting (June 24-25, 1989), Vienna, Austria.

7. Trirawatanapong, T., and Padmanabhan, R. Identification of a neutralizing epitope on the envelope glycoprotein of dengue virus type 2. Presented at the Second International Symposium on Positive Strand RNA Viruses held at Vienna, Austria (June 26-30, 1989).

8. Trirawatanapong, T. and Padmanabhan, R. Identification of a neutralizing epitope of envelope glycoprotein (E) of dengue virus type 2. Presented at the 1989 meeting on MODERN APPROACHES TO NEW VACCINES Including Prevention of AIDS, held at the Cold Spring Harbor Laboratory between Sept. 20-24, 1989.

7.0 List of Personnel received Contract Support

Personnel	Period of Support
Research Assistants	
Dianne Vassmer	Sept. 1986-Feb. 1988
Karine Page	Nov. 1986-Oct. 1987
Kevin Graham	March 1988-Aug. 1990
Melissa Larson	June, 1988-Sept. 1988
Yifan Xu	Feb. 1989-June 1989
Qing Xhu-Zhang	Nov. 1990-April 1991
Graduate Students	
Thaweesak Trirawatanapong	Sept. 1986-July 1990
Luwen Zhang	Sept. 1988-April 1991
Raghuram Kalluri	Sept. 1988-March 1989
Post-doctoral Research Associates	
Dr. Yasuyuki Sasaguri	Sept. 1986-July 1987
Dr. Kamal Bittar	Sept. 1986-Dec. 1986
Dr. Gunwar Sripad	Nov. 1986-Oct. 1987

Dr. Maruthi Mohan	May 1987-March 1989
Dr. Koji Irie	Jan. 1987- Dec. 1988
Dr. Akihiko Nakashima	March 1988-June 1988
Dr. Shahana Saroya	Oct. 1990-April 1991

Laboratory Aides

L.R. Anderson (part-time)	Sept. 1986-Sept. 1987
Tina Nguyen (part-time)	Sept. 1986-Sept. 1987
Cindy Smith (part-time)	Sept. 1986-May 1988
Anthony Martinez (part-time)	April 1988-Sept. 1988

7.1 Ph.D. degree conferred

Thaweesak Trirawatanapong	July 1990